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Effects of avenanthramides on oxidant generation and antioxidant enzyme activity in exercised rats

Li Li Ji[a,b,*], David Lay[a], Eunhee Chung[a], Ying Fu[a], David M. Peterson[c]

[a]*Department of Kinesiology, University of Wisconsin-Madison, 2000 Observatory Drive, Madison, WI, 53706*
[b]*Division of Nutritional Science, University of Wisconsin-Madison, 2000 Observatory Drive, Madison, WI, 53706*
[c]*USDA, Agriculture Research Service, Cereal Crops Research Unit, Madison, WI 53726*

Abstract

Avenanthramides (AVEN) are major phenolic acids in oat (*Avena sativa* L.) that have potent antioxidant functions. Female Sprague-Dawley rats (n = 48, age 6-7 wk) were fed either an AIN-93 based control (C) diet or the same diet containing 0.1 g/kg AVEN-Bc [*N*-(3',4'-dihydroxycinnamoyl)-5-hydroxyanthranilic acid] for 50 days. Each group was further divided into rested (R) and exercised (E, treadmill running at 22.5 m/min, 10% grade for 1 hour) prior to killing. AVEN supplementation per se had no effect on reactive oxygen species (ROS) production in most tissues, except soleus muscle wherein ROS level was decreased. AVEN-fed rats had higher superoxide dismutase (SOD) activity in the deep portion of vastus lateralis muscle (DVL), liver and kidney and higher glutathione peroxidase (GPX) activity in the heart and DVL, compared to C-fed rats. E increased ROS production in the liver, DVL and soleus, and lipid peroxidation in the heart, liver and DVL. AVEN attenuated E-induced ROS in soleus and lipid peroxidation in the heart, but enhanced lipid peroxidation in DVL. We conclude that AVEN can serve as a potential dietary antioxidant supplement, but its tissue specific effects require further investigation. © 2003 Elsevier Inc. All rights reserved.

Keywords: Antioxidant; Avenanthramide; Exercise; Lipid peroxidation; Rat; Reactive oxygen species

* Corresponding author: Tel.: +1-608-262-7250; fax: +1-608-262-1656.
E-mail address: ji@education.wisc.edu (L.L. Ji).

1. Introduction

Reactive oxygen species (ROS) are generated ubiquitously in aerobic organisms [1]. When these cytotoxic agents overwhelm endogenous antioxidant defense systems, serious oxidative stress and damage occur as reflected by the oxidative modification of macromolecules such as lipid, protein and DNA [2]. ROS generation has an important implication in the etiology of many diseases and in aging [3,4]. Furthermore, strenuous physical exercise has been shown to increase oxidative stress to skeletal muscle and myocardium due to increased ROS generation [5]. Thus, one of the important tasks is to seek novel antioxidants that can enhance endogenous defenses against oxidative damage. Dietary supplementation and therapeutic use of antioxidants, such as those present in fruits and vegetables known as phytochemicals, are emerging measures in nutritional biochemistry [6–8].

Oats (*Avena sativa* L.) contain several families of phytochemicals that display antioxidant properties, such as tocotrienols, phenolic acids, flavonoids, sterols and phytic acid [9–11]. Both animal studies and human clinical trials confirmed that oat antioxidants have the potential of reducing cardiovascular risks by lowering serum cholesterol, inhibiting LDL oxidation, and attenuating platelet aggregation and peroxidation [12]. In vivo animal studies also have shown that caffeic and ferulic acids possess anti-carcinogenic properties [13]. In addition to these well-characterized antioxidants, there is a small fraction of anionic, nitrogen-containing, covalently linked hydroxycinnamic acid compounds that have only been identified in oats, called avenanthramides (AVEN) [14,15]. Among a group of several avenanthramides that differ in the substituents on the cinnamic acid and anthranilic acid rings, three are predominant in oat grain: Bp, Bf and Bc [16]. In vitro experiments indicate that they have significant antioxidant activities, with Bc >Bf> Bp [16,17], whereas little is known about their in vivo health benefits [15].

Our overall hypothesis, therefore, is that dietary AVEN supplementation increases antioxidant capacity in the biological tissues, thereby reducing steady-state ROS formation and oxidative tissue damage. In order to reveal their potential protective effects, we subjected AVEN-fed rats to an acute bout of strenuous physical exercise, which is known to increase ROS generation in the heart and skeletal muscle [5,18]. Our specific hypotheses are: (1) dietary AVEN supplementation attenuates exercise-induced ROS generation and oxidative tissue damage, and (2) AVEN supplementation increases endogenous antioxidant enzyme activities in the various tissues of rats.

2. Methods and materials

2.1. Animals

Female Sprague-Dawley rats (n = 48, age 6-7 wk) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and housed two per cage in a temperature controlled room (22°C) with a 12-12 h dark-light cycle (8:00-20:00 light; 20:00-8:00 dark) at the Animal Science Facilities of the University of Wisconsin-Madison. Female rats were used because

Table 1
Composition of the Diet

Ingredient	Diet g/kg diet
Casein (84% protein)	200.0
L-Cystein	3.0
Cornstarch	397.886
Maltodextrin[1]	132.0
Sucrose	100.0
Soybean oil	70.0
Cellulose[2]	49.5
Choline bitartrate	2.5
D,L-Methionine	0.30
Mineral Mixture[3], AIN-93G-MX	35.0
Vitamin Mixture[3], AIN-93-VX	10.0
TBHQ[4] (antioxidant)	0.014
Avenanthramide-Bc	0.1

[1] Dextrinized corn starch (hydrolysate, 90-94% tetrasaccharides and higher)

[2] Solka-Floc[R], 200 FCC.

[3] AIN-93G-MX, mineral mix; AIN-93-VX, vitamin mix, recommended by American Institute of Nutrition (AIN) (J. Nutr. 123:1939-1951, 1993.)

[4] tert-butylhydroquinone.

male rats are known to decrease their food intake during exercise training on treadmill, thereby confounding the results due to decreased antioxidant consumption. Female rats, on the other hand, maintain food intake and body weight during training. Before the experiments began, rats were monitored daily and had free access to water and a chow diet. The experimental protocol was approved by the University of Wisconsin Research Animal Resources Center.

2.2. Avenanthramide synthesis

Avenanthramide Bc [*N*-(3',4'-dihydroxycinnamoyl)-5-hydroxyanthranilic acid] was synthesized from caffeic acid and 5-hydroxyanthranilic acid as described previously [17]. The reaction products were chromatographed on a Sephadex LH-20 column, and the fractions containing Bc were combined. The purity was verified by HPLC retention time and UV spectra. The compound was shown to be identical to Bc extracted from oat grains by ^{1}H NMR spectroscopy.

2.3. Avenathramide supplementation

After two days of acclimation the rats were randomly divided into two dietary groups and housed in individual cages. Both groups were fed an AIN-93 based purified diet (Table 1) except that one diet contained 0.1 g/kg avenathramide-Bc, whereas the other contained an equal amount of starch. This level of supplementation provided the same amount of AVEN that could be obtained by rats consuming a diet consisting of 50-100% oats. The diets were

manufactured by Harlan-Teklad Co. (Madison, WI). Each group of rats was fed the respective diet for 50 days, before they were randomly divided into exercised (E) and rested (R) treatment groups.

2.4. Exercise

On the day of experiment, the rats from the E group were subjected to an acute bout of running on a Quinton rodent treadmill at 22.5 m/min, 10% grade for 1 hour. This intensity represents approximately 75% of VO_2max for untrained rats at this age, according to Brooks and White [19]. The rats of the R group were rested in their cages without access to food or water for the same amount of time.

2.5. Tissue preparation

Immediately after the acute exercise bout or the 1-hour rest period, the rats were decapitated followed by exanguination. The liver, heart, kidney, the deep portion of vastus lateralis (DVL) muscle and the soleus muscle from both hind legs were quickly removed. A portion of each of the various tissues was submerged in an ice-cold buffer medium containing 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris-HCl, and 30 mM glucose (pH 7.4), plus 2 mM malate and 2 mM pyruvate. The remaining portions of the tissues were freeze-clamped in brass tongs pre-cooled in liquid nitrogen. Immediately, these various tissues in the buffer media were minced thoroughly and homogenized with a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C at low speed. DVL homogenate was filtered through four layers of medical gauze to remove connective tissue debris. An aliquot of each homogenate was taken for oxidant measurement (see below). The remaining homogenates and frozen tissues were stored at −80°C for other biochemical analyses.

2.6. Determination of oxidant production

Generation of muscle oxidant was evaluated using 2',7'-dichlorofluorescein (DCFH) as a probe according to LeBel and Bondy [20] with modifications [21,22]. The DCFH assay is sensitive to both ROS and reactive nitrogen species (RNS) in the defined assay conditions due to the existence of NO synthase in muscle [23]. The assay medium contained 5.0 μM DCFH-diacetate dissolved in 1.25 mM methanol and an appropriate amount of homogenate, and was incubated at 37°C for 15 min. DCF formation was followed for 30 min at the excitation wavelength of 488 nm and emission wavelength of 525 nm using a Hitachi F-2000 fluorescence spectrometer. The rates of DCFH conversion to DCF, which were linear for at least 60 min, were corrected with the autooxidation rate of DCFH without protein. All assays were carried out in duplicate. The unit was pmol DCF formed $\times$ min^{-1} $\times$ mg protein^{-1}.

2.7. Antioxidant enzymes

Total SOD (EC 1.15.1.1) activities in homogenates from the various tissues were measured according to Sun and Zigman [24]. Glutathione peroxidase (GPX, EC 1.11.1.9) activity

was measured by the method of Flohe and Gunzler [25]. Protein content was measured by the Bradford method using bovine serum albumin as a standard.

2.8. Lipid peroxidation

Peroxidative damage to cellular lipid constituents was determined by measuring malondialdehyde (MDA) in butanol extracts spectrophometrically according to Uchiyama and Mihara [26] with modifications as follows: 10 mM butylated hydroxytoluene and 200 mM ferrous sulfate were included in the assay mixture. Sealed tubes were incubated for 15 min at 99°C. MDA content was calculated based on a standard curve using 1,1,3,3-tetraethoxypropane as a standard.

2.9. Statistical analysis

The data were analyzed using a two-way ANOVA. After a significant treatment effect (age, exercise, or interaction) was found, Shaffè's Post hoc tests were performed to determine the significance among means. $P < 0.05$ was considered significant.

3. Results

Body weight of the rats was not different between the treatment groups at the beginning of the experiment. Body weight for each treatment group in the end of the experiment was as follows: control rested, 226 ± 2.9 g; AVEN rested, 224 ± 2.9 g; control exercised, 224 ± 2.9 g; AVEN exercised, 225 ± 2.9. There was no significant difference among the various groups. Records of food consumption during the 50-day feeding period showed no difference between the two groups of rats. Daily food consumption was approximately 7 g/100 g body wt for both groups in the end of the experimental period. Estimated AVEN consumption was thus ~0.7 mg/100 g body wt for AVEN-supplemented rats.

AVEN supplementation did not affect ROS concentration in the liver (Fig. 1a). E rats had significantly higher ROS levels in the liver than R rats ($P < 0.05$) in both dietary groups. In the heart (Fig. 1b) there was a significant interaction between AVEN and E ($P < 0.05$). Among the exercised groups of rats, those whose diet was supplemented with AVEN had a greater ROS concentration than C rats ($P < 0.05$), whereas no difference was found between rested rats fed the two experimental diets. ROS in the kidney was not affected by either AVEN or E (Fig. 1c). Exercise increased ROS generation in two types of skeletal muscle. In DVL, there was a trend towards a higher ROS concentration in E vs. R rats ($0.05 < P < 0.1$), and AVEN did not influence ROS levels. In soleus muscle, E rats had significantly high ROS levels than R rats ($P < 0.05$). AVEN decreased ROS generation ($P < 0.05$) and attenuated exercise induced ROS ($P < 0.05$) (Fig. 1e).

AVEN supplementation increased SOD activity in the liver, kidney, DVL and soleus muscle ($P < 0.01$) (Fig. 2). In the heart, however, AVEN-supplemented exercised rats showed significantly lower SOD activity than any other group ($P < 0.05$, diet × exercise interaction).
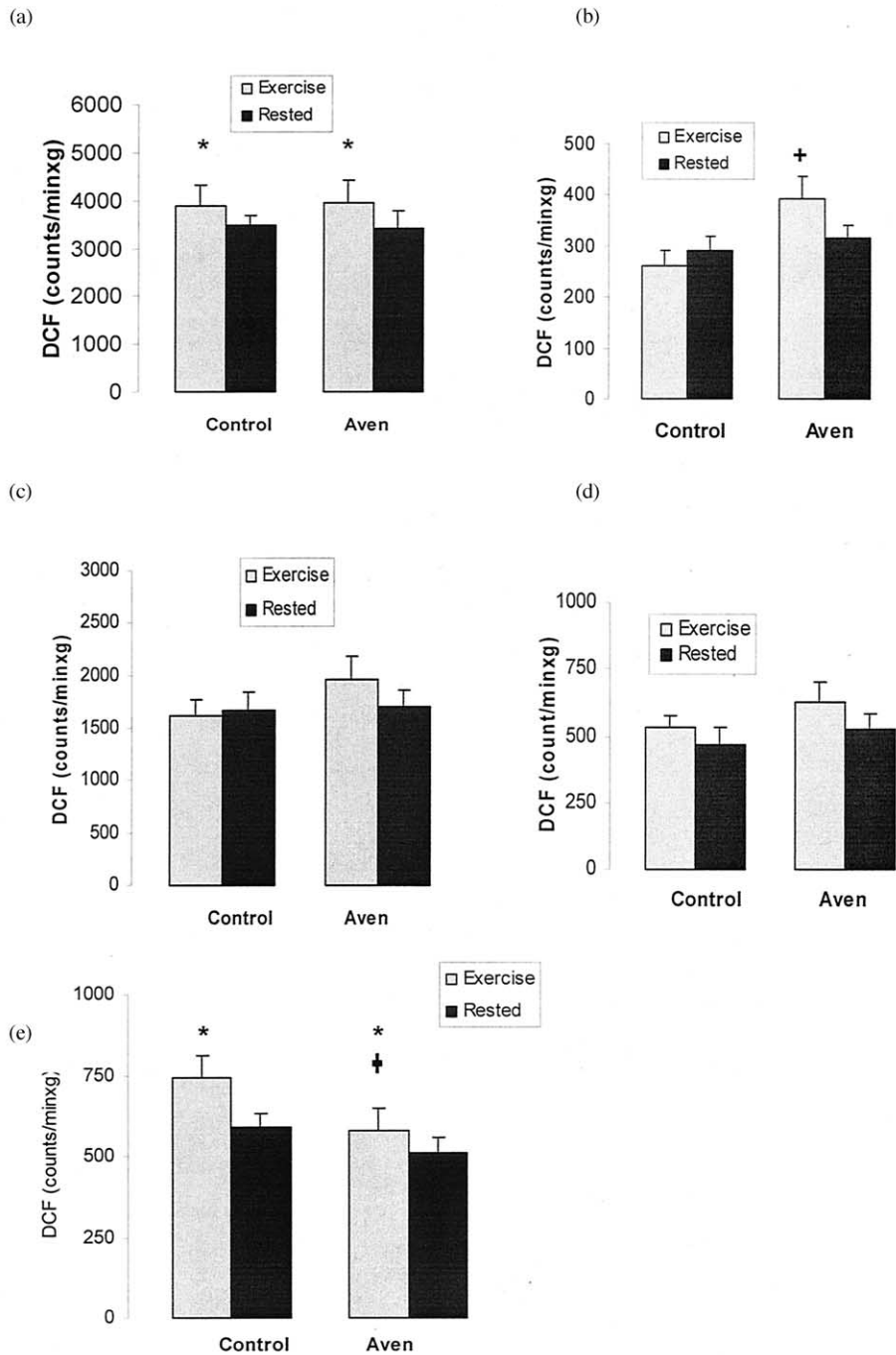
Fig. 1. Oxidation rate of dichlorofluorescin (DCFH) to dichlorofluorescein (DCF) in the homogenates of liver (a), heart (b), kidney (c), deep vastus lateralis (DVL) muscle (d), and soleus muscle (e) of avenanthramide (Aven) supplemented and control rats. The assay buffer contained 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris-HCl, and 30 mM glucose (pH 7.4), plus 2 mM malate and 2 mM pyruvate. Each bar represents mean ± SEM (n=12). *$P<0.05$, Exercised vs. Rested. [+] $P<0.05$, Aven vs. Controls.
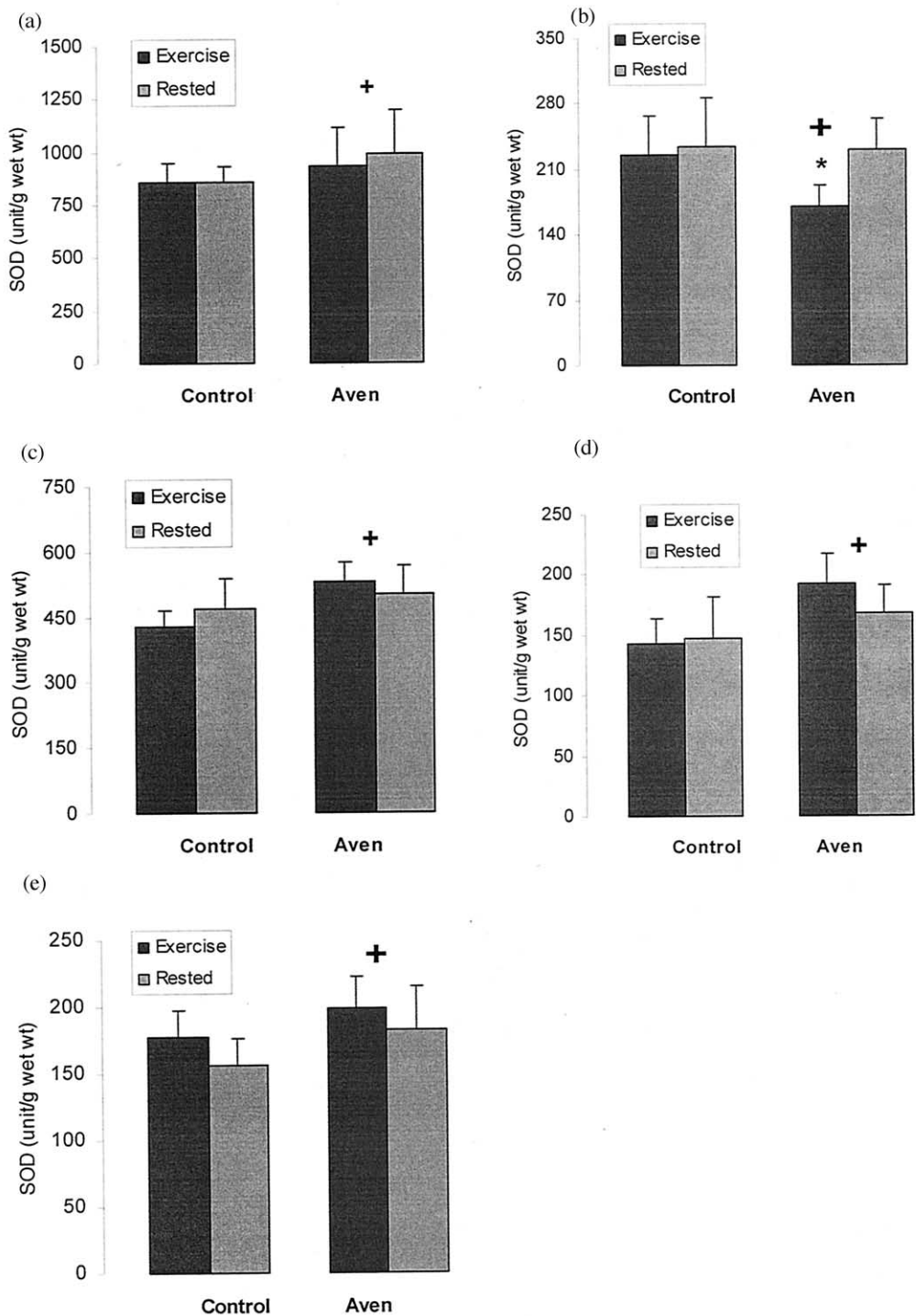
Fig. 2. Superoxide dismutase (SOD) activity in the liver (a), heart (b), kidney (c), deep vastus lateralis (DVL) muscle (d), and soleus muscle (e) of avenanthramide (Aven) supplemented and control rats. Each bar represents mean ± SEM (n = 12). *P < 0.05, Exercised vs. Rested. [+]P < 0.05, Aven vs. Controls.
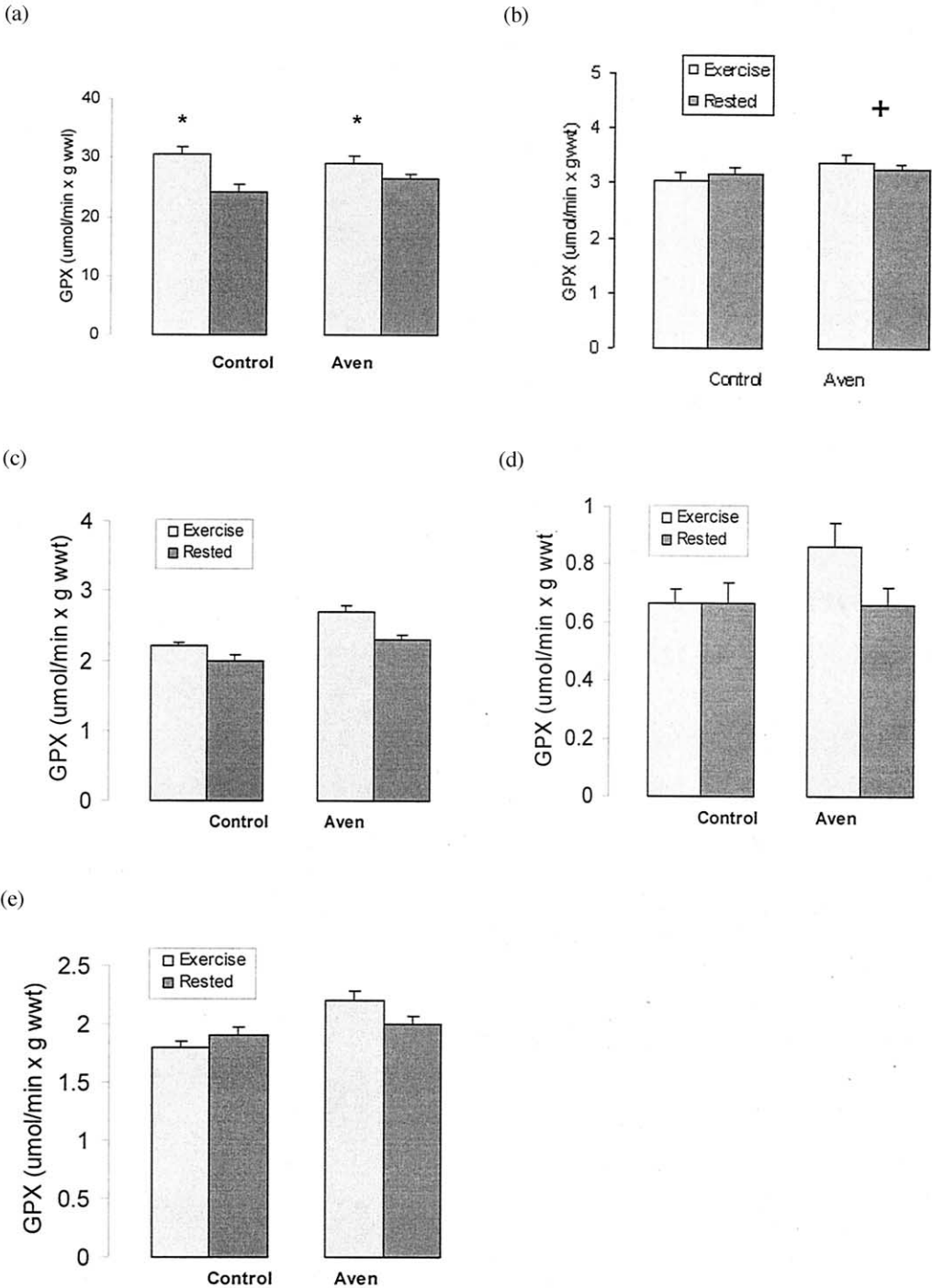
Fig. 3. Glutathione peroxidase (GPX) activity in the liver (a), heart (b), kidney (c), deep vastus lateralis (DVL) muscle (d), and soleus muscle (e) of avenanthramide (Aven) supplemented and control rats. Each bar represents mean $\pm$ SEM (n=12). *$P < 0.05$, Exercised vs. Rested. +$P < 0.05$, Aven vs. Controls.
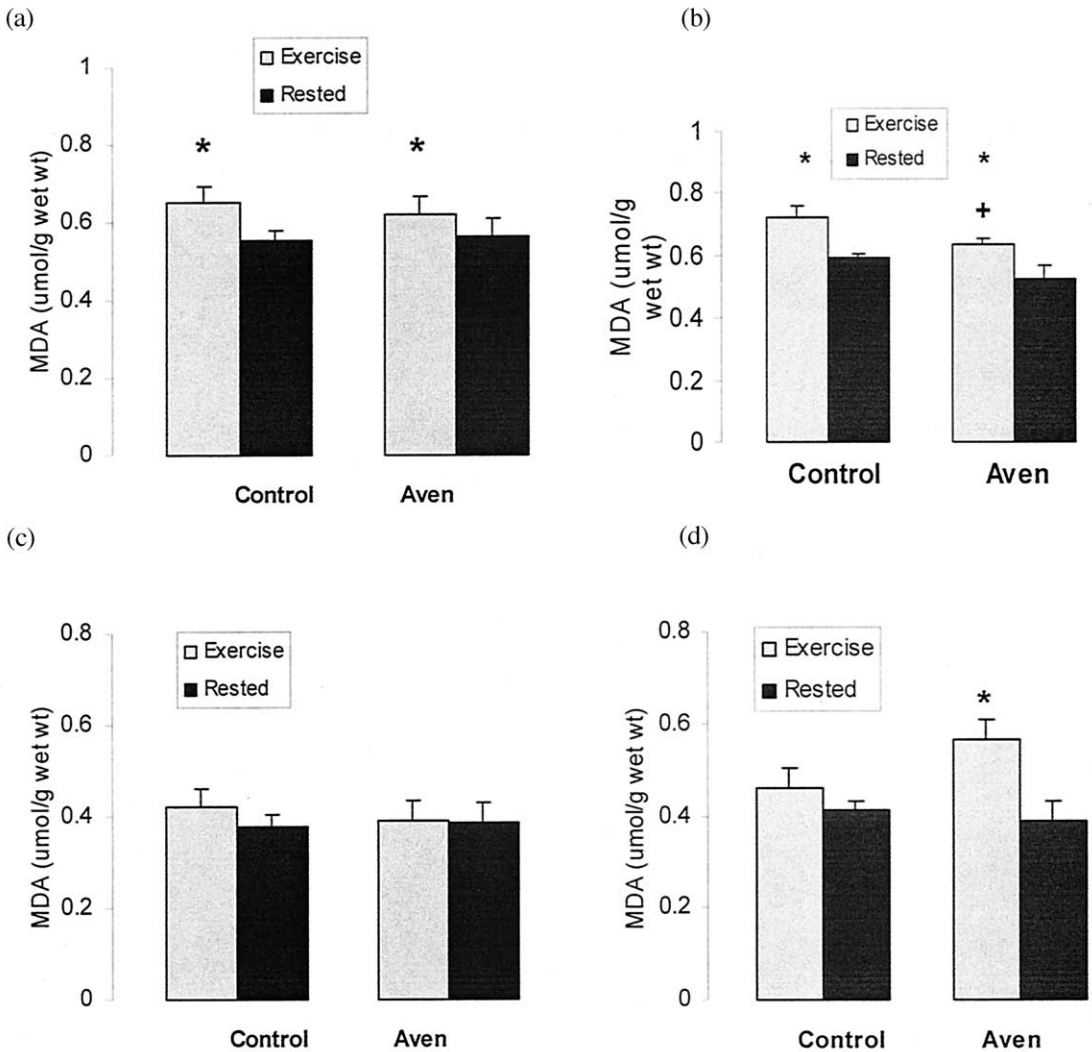
Fig. 4. Malondialdehyde (MDA) content in the liver (a), heart (b), kidney (c), and deep vastus lateralis (DVL) muscle (d) of avenanthramide (Aven) supplemented and control rats. Each bar represents mean $\pm$ SEM (n = 12). *P $<$ 0.05, Exercised vs. Rested. [+]P $<$ 0.05, Aven vs. Controls.

GPX activity in the liver was increased in E vs. R rats (P $<$ 0.05), but unaffected by AVEN treatment (Fig. 3a). GPX activity tended to be higher (P $<$ 0.09) in the heart of AVEN-treated rats (Fig. 3b). In the kidney, DVL and soleus, GPX activity was not altered with either AVEN or exercise.

Tissue lipid peroxidation using MDA content as a marker was elevated in the liver (P $<$ 0.05), heart (P $<$ 0.01) and DVL (P $<$ 0.05) of E vs. R rats (Fig. 4). AVEN supplementation did not influence MDA levels in the liver or DVL, but decreased exercise-induced lipid peroxidation in the heart (P $<$ 0.05).

4. Discussion

Oat avenanthramides have demonstrated potent antioxidant properties in vitro. However, their protection against in vivo oxidative tissue damage has not been studied. In the present study, we demonstrated that dietary supplementation of a synthetic avenanthramide (Bc) had altered intracellular oxidant-antioxidant balance in rats. Furthermore, AVEN demonstrated potential antioxidant function against exercise-induced oxidative stress in selected tissues.

AVEN administration did not change the rate of resting ROS generation in most tissues measured. This finding suggests that this synthetic antioxidant did not influence cellular metabolism for energy derivation. At rest, mitochondria are the main source of ROS production as a result of the electron "leakage" through the respiratory chain [27]. Several membrane-borne and cytosolic oxidases, such as NADPH oxidase, cycloxygenase and xanthine oxidase, as well as peroxisomes, may also contribute to basal ROS generation, albeit of minor importance [2]. ROS generation, however, was significantly increased in liver, soleus and DVL ($P < 0.1$) muscle after an acute bout of exercise, a finding consistent with previous studies [5,21]. AVEN treatment attenuated exercise-induced ROS in soleus, a predominately slow-twitch oxidative muscle recruited in endurance exercise, but not in DVL muscle. This fiber-specific effect of AVEN on muscle ROS product may be related to the difference of capillary density and rate of blood perfusion, as these profiles may affect deposition and cell concentration of AVEN after chronic supplementation. Interestingly, in the heart ROS production was increased after the acute exercise bout only in AVEN-supplemented rats. This specific effect of AVEN cannot be readily explained, but may be related to the alteration of blood flow to the various organs during exercise, which may affect the AVEN concentration in the tissue and its interaction with ROS. While it is well-known that certain phenolic compounds can exhibit pro-oxidant action, such as catechins, when present in high concentration [28], the magnitude of required concentrations (usually in mM range) are not likely achievable in the heart in an in vivo experiment. However, we currently have no data on the bioavailability, distribution and tissue concentrations of AVEN. More careful research is needed to delineate these characteristics in response to an oral supplementation regimen and exercise.

AVEN administration increased SOD activity in all tissues measured except the heart. Inversely, only heart showed higher GPX activity as a result of AVEN treatment. These findings are significant as they suggest that AVEN may not only act as a ROS scavenger, but may also induce endogenous antioxidant enzymes. It is not clear, however, whether the increased enzyme activity was due to an activation of the existing enzyme molecules or an upregulation of gene expression. Given the SOD assay condition wherein an in vivo superoxide anion ($O_2^{-\cdot}$) generating system (epinephrine autooxidation) was used, it is possible that increased tissue AVEN content provided a $O_2^{-\cdot}$ "sink", creating a psuedo SOD activity. The observation that ROS generation was higher in the heart of AVEN-treated exercise rats, whereas SOD activity was lower, appear to add some merit to this speculation. However, this speculation would also imply that chronic supplementation of AVEN had significantly elevated AVEN levels in most body tissues except heart. Future studies measuring both tissue AVEN level and SOD enzyme protein content would aid to clarifying this controversy.

Lipid peroxidation occurs when ROS attack membrane polyunsaturated fatty acids, leading to their disintegration and formation of short-chain alkyl radicals and aldehydes. Lipid soluble antioxidants such as α-tocopherol are known to scavenge ROS and prevent the free radical chain reaction. In several previous studies, animals fed an oat-supplemented diet showed decreased lipid peroxidation in carcass meat compared to those fed a control diet, although species difference exist [29,30]. In the current study, AVEN supplementation per se had no effect on resting MDA levels in several tissues, possibly due to the short feeding regimen. However, AVEN supplementation attenuated lipid peroxidation induced by an acute bout of exercise in the heart. This finding suggests that AVEN may have antioxidant effects in specific tissues. In the heart, exercise increased ROS production and decreased SOD activity in AVEN-treated rats. As a result, GPX activity was elevated, which could aid in the removal of lipid peroxide, the precursor for MDA. Such compensatory mechanism may not be present in other tissues. These tissue specific-effects of AVEN require further investigation in order to fully understand its antioxidant potential and utility under physiological conditions.

In summary, we have reported here for the first time that dietary supplementation of a synthetic avenanthramide selectively attenuated exercise-induced ROS production and lipid peoxidation. The antioxidant effects of AVEN were likely related to its ability to influence tissue antioxidant enzyme systems such as SOD and GPX activities.

Acknowledgments

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